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ORIGINAL ARTICLE

Isolation and characterization of potential probiotic yeasts from Ethiopian injera sourdough

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Abstract

This study aimed to isolate and characterize potential probiotic yeasts from Ethiopian injera sourdough and the study was conducted by collecting samples from Gondar and Bahir Dar cities, Ethiopia. The potential yeasts were isolated and identified using morphological, physiological, biochemical and molecular based analysis. Promising isolates were selected to further investigate their in vitro probiotic properties, including survival at different temperatures (25, 30, 37, and 42 °C), acidic pH (2, 3, 4 and 5), bile salt (0.1, 0.3, and 0.5%), and osmotolerance (20, 30, 40, and 50% glucose concentration), antimicrobial activities, proteolytic and lipolytic activities as well as resistance to four antibiotics. From 20 samples, 38 isolates were obtained. Among these, 10 produced low or non-hydrogen sulfide and were selected for further work. Further screening tests revealed that five isolates (G1N1, G2N4, G3N1, G8N1, and B6N3) were able tolerate and grow at 37 °C, with harsh conditions of the human digestive tract like low pH, bile salt, and higher osmotic effect. The maximum growth OD values were recorded at 37 °C by isolate G4N1 (OD value (0.6667), while G3N1 exhibited a maximum growth OD value of 0.4227 at pH 2. On the other hand, G2N4 gave a maximum OD value of 0.8800 at 0.3% bile salt concentration. The promising isolates were sequenced and identified to species level. Based on phylogenetic tree analysis, all the five probiotic yeast isolates had one common ancestor and belonging to *Saccharomyces cerevisiae* (G1N1 and G2N4), *Candida humilis* (G3N1 and B6N3), and *Pichia kudriavzevii* (G8N1). This study revealed that Ethiopian injera sourdough could be potential source of different probiotic yeast strains. Strong emphasis should be given about the use of probiotic yeasts that are isolated from Ethiopian fermented foods.

Keywords Fermented food · Injera sourdough · Probiotic yeast · *S. cerevisiae* var. *boulardii*

Introduction

Fermentation is one of the oldest food processing and preservation technologies which was/is practiced in the different populations of the world including Ethiopians, with distinct microbial populations. Ethiopians consume a wide range of traditional fermented foods, and beverages that are made from a varied range of basic raw materials, the majority of which are spontaneously fermented products like injera, kocho, ergo, ititu, tella, tej, borde, awaze, etc., have been prepared and consumed by Ethiopian communities (Kindu 2019). The major role played in fermenting Ethiopian traditional foods including injera from sourdough is by yeasts. Injera is a thin, soft fermented baked food made from flour of cereals such as teff (*Eragrostis teff*)—dominantly used source (Barretto et al. 2021), sorghum, teff, corn, wheat, and barley (Satheesh and Solomon 2020).

Probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts (Salminen et al. 2005). The demand of probiotic functional foods is growing from time to time due to increased awareness of consumers about the impact of food on health (Tripathi and Giri 2014). Probiotic yeasts such as *S. cerevisiae* var. *boulardii* and various yeast strains improve colon immune status and aid in the prevention and reduction of pathogenic and infectious bacteria in the gastrointestinal (GI) tract (Kunyeit et al. 2020). Furthermore, probiotic yeasts are useful in the treatment of lactose intolerance and diarrhea (Mezemir 2015).

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Probiotics can grow at 37 °C, withstand the harsh conditions of the human digestive tract such as digestive enzymes, pancreatic juice, bile salt, and stomach acid (low pH) (Sarwar et al. 2019), and contribute to the host environment's health by regulating microbiota and performing biological functions. Some probiotic yeasts also adhere to gut epithelial cells and mucus to compete with pathogenic microorganisms (Staniszewski and Kordowska-Wiater 2021). *S. cerevisiae* var. *boulardii* is a unique probiotic yeast that has an exceptionally high optimal development temperature of 37 °C, and known to survive gastric acidity, and it is not adversely affected or inhibited by antibiotics or does not alter or adversely affect the normal microbiota in the bowel (Corbo et al. 2017). These probiotics can be consumed as food ingredients or non-food supplements (Mezemir 2015).

The reported beneficial effects of probiotic consumption include improvement of intestinal health, enhancement of the immune response, reduction of serum cholesterol, and cancer prevention (Salminen et al. 2005). Health benefits have been predominantly demonstrated for specific probiotic strains of the bacteria genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Bacillus* (Staniszewski and Kordowska-Wiater 2021), while one strain of yeast, *S. cerevisiae* var. *boulardii*, has been found to be an effective probiotic in double-blind clinical studies (Czerucka et al. 2007) and there is a need to further study in detail about this yeast and other potential ones. And also increasing multidrug resistant pathogens, increased demand for natural drug substitutes, and the emergence of scientific and clinical evidences demonstrating the efficacy of probiotics have prompted researchers to investigate probiotics as alternative remedies (Khagwal et al. 2019).

As to the best of our knowledge, isolation, identification, and molecular characterization of probiotic yeasts from Ethiopian injera sourdough are not studied. The physical and chemical nature of these probiotic yeasts is not fully investigated. In addition, the antagonistic effect of probiotic yeasts against gut microbes and their degree of efficacy on pathogenic microbes has not been determined. Therefore, the current study is aimed to isolate and characterize potential probiotic yeasts under local setting from injera sourdough.

Materials and methods

Study area

This study was conducted by collecting injera sourdough samples from Gondar and Bahir Dar cities of Amhara region, Ethiopia. Gondar is located in northwestern part of Ethiopia, Amhara region. It is about 727 km away from Addis Ababa (the capital city of Ethiopia). It is located at 12° 3′ north latitude and 37° 28′ east longitude. The city has an altitude of 2133 m above sea level. The annual precipitation and temperature averages of the study area are 1161 mm and 26 °C, respectively. Gondar city consists of a total of 50, 817 housing units (Genetie 2020).

Bahir Dar is the capital city of Amhara region in northwestern part of Ethiopia, and the city is located approximately 578 km distant from Addis Ababa, the capital city of Ethiopia. It is located at 11° 36′ north latitude and 37° 23′ east longitude and at an altitude of 1820 m above sea level. Its mean annual temperature is 27 °C, and the annual precipitation ranges from 1200 to 1600 mm. The two study sites were selected purposively as they are major injera producing towns in Amhara region of Ethiopia.

Sample size and sampling technique

Ten samples were collected randomly from each Gondar and Bahir Dar cities summing up a total of twenty injera sourdough samples. About 10 ml injera sourdough samples were collected in each case. The collected samples were placed aseptically in sterile labeled falcon tubes. Then, the tubes were transported to Microbiology Laboratory, Institute of Biotechnology, University of Gondar. All samples were kept at 4 °C until used.

Isolation of yeasts and cultural characteristics

Injera sourdough samples were serially diluted (10^{-1} to 10^{-5}) using peptone water as a diluent by taking one ml of each sample transferred to nine ml of peptone water in test tubes and mixed thoroughly. A volume of 0.1 ml of suspension from these serially diluted tubes were taken and spread evenly with a sterile L-shaped glass rod over the surface of a sterilized yeast extract peptone dextrose agar (YPDA) media containing yeast extract, 5 g/l; peptone, 5 g/l; D-glucose, 10 g/l and 20 g/l agar was used and adjusted to pH 5 before autoclaving and 0.5 µg/l chloramphenicol. Then, the inoculated agar plates were incubated at 30 °C for 48 h. Based on colony shape, elevation, edge/margin, colony size, colony color, and surface properties of representative colonies which showed comparatively different cultural characteristics were sub-cultured onto different YPDA plates so as to obtain the pure culture of each likely different colony. Thereafter, each separately grown colony was examined for cultural and morphological characteristics according to the method of Teramoto et al. (2005) and maintained using YPDA media for further use.

Identification of yeast isolates

Identification of yeast isolates to the species level was carried out based on morphological, physiological, biochemical tests as well as molecular based analysis.

Microscopic morphological characterization of yeast isolates

To determine the morphology of yeast cells and reproduction type, the cultures were examined microscopically. Vegetative cells were observed after 3 days of incubation in YPD broth adjusted to pH 5 at 30 °C. Sample from each yeast was smeared and then stained with diluted methylene blue and observed under a light microscope at (X100) magnification using oil immersion objective (Barnett et al. 2000; Yuma 2020).

Hydrogen sulfide production test

To test whether each yeast isolate produces hydrogen sulfide or not, an approach used by Tsegaye et al. (2018) was adopted. Bismuth sulfite agar medium (20 g of BSA suspended in 500 ml of distilled water) was used to examine the production of hydrogen sulfide. Each yeast isolate was streaked onto a separate BSA plates using wire loop and incubated at 37 °C for 2 to 5 days. Isolates, which presented light to dark black colony color, were regarded as hydrogen sulfide producers, whereas isolates with non-black colonies were identified as non-producers (Tsegaye et al. 2018).

Physiological characterization of probiotic yeast isolates

Potential probiotic yeast isolates were screened using physiological characterization tests. For undertaking physiological characterization tests, each pre-grown yeast culture was adjusted to an absorbance value of 0.5 McFarland standards which correspond to a concentration of 10^8 cell/ml (Antia et al. 2018).

Temperature tolerance test

The growth of yeast isolates was evaluated at varying temperatures (Antia et al. 2018). About 1 ml of fresh yeast culture was standardized to 10^8 cells/ml and inoculated separately into 40 ml YPD broth and adjusted to pH 5 before autoclaving. The inoculated test tubes were incubated at 25, 30, 37, and 42 °C temperatures. The growth of each yeast isolate was measured using spectrophotometer at 600 nm with intervals of 24, 48, and 72 h. Non-inoculated YPD broth was used as blank solution (Antia et al. 2018; Truong et al. 2021).

Bile salt tolerance test

To check bile salt tolerance test, the tubes containing YPD medium were supplemented with different concentrations of bile salt (0.1, 0.3, and 0.5%) and were inoculated with 1 ml (10^8 cfu/ml) of yeast cells. The bile salt tolerance of isolates was evaluated based on estimating growth of the yeast cells after 48 h of incubation at 37ºC by measuring their absorbance at 600 nm (Bhukya et al. 2010; Katarzyna and Alina 2010).

pH tolerance test

Growth of yeast isolates at low pH (acidic environment) was determined using the method of Rajkowska and Kunicka (2010) with some modifications of pH range. The pH of sterile 40 ml YPD broth was adjusted to 2, 3, 4, and 5 pH with 3 M HCl and NaOH. The broth media was inoculated separately with 1 ml (10^8cells/ml) fresh yeast isolate culture. They were incubated at 37 °C for 72 h. The pH tolerance and growth of the yeast isolates were examined using spectrophotometer at optical density of 600 nm with intervals of 24, 48, and 72 h. YPD broth without yeast isolate was used as blank solution (Rajkowska and Kunicka 2010; Antia et al. 2018).

Osmotolerance test

The osmotic tolerance of each yeast isolate was also evaluated (Karki et al. 2017). YPD broth media containing 20, 30, 40, and 50% glucose separately and adjusted to optimize pH were dispensed into screw caped test tubes and sterilized. About 1 ml of each yeast isolate (10^8 cells/ ml) was inoculated separately into 40 ml YPD broth and was incubated at 37 °C for 72 h. Growth of each yeast isolate was determined by measuring the optical densities of the respective culture separately at 600 nm using a spectrophotometer after 24, 48, and 72 h. YPD broth medium containing respective glucose concentration alone was used as blank solution (Karki et al. 2017).

Biochemical characterization of probiotic yeasts

Carbohydrate fermentation test

Carbohydrate fermentation test (glucose, sucrose, galactose, maltose, and lactose) was performed (Barnett et al. 2000). The fermentation capacity of each carbohydrate by each yeast isolate was estimated via observing media color change followed by the formation of gas or not in the 5 ml Durham tube at the incubation. About 40 ml of carbohydrate test medium containing 10% of those test sugars, yeast extract, 5 g/l; peptone, 5 g/l; 2–3 drops of phenol red

and adjusted to optimized pH was dispensed into test tubes with an inverted Durham's tubes and sterilized at 121 °C for 15 min. Then, 0.1 ml of each yeast isolate that was pre-grown on YPD broth medium and standardized to 10^8 cells/ml was inoculated separately into each test tube and incubated at 30 °C for 48 h. After incubation, each tube and the inverted Durham tube within it were observed for gas formation and media color change as result of acid production (Barnett et al.2000).

Proteolytic activity test

To determine the proteolytic activity of strains, each yeast isolate was streaked on skim milk agar medium using wire loop. The inoculated agar plates were incubated at 37 °C for 72 h. Colonies having clearance (halo) surrounding them were considered as strains with proteolytic activity (Kivanc and Yapici 2015).

Lipolytic activity test

Using wire loop, each yeast isolate was streaked on tween 80 media (10 ml/l tween 80, 10 g/l peptone, 5 g/l sodium chloride, 0.1 g/l calcium chloride, and 20 g/l agar) on a separate plate and incubated at 37 °C for 2 to 5 days. For lipolytic activities of strains, isolates showed scattered form of growth (precipitation), as the calcium salt, of the fatty acid released by hydrolysis of tween 80 yeast colonies around streak inoculated media considered as lipolytic isolates (Plou et al. 1998).

Hemolytic activity test

The hemolytic activity assay was carried out to evaluate the pathogenicity of each yeast isolate which could serve as the criteria for choosing a probiotic strain. The strains were tested for hemolytic activity using blood agar (5% v/v sheep blood, 0.5% peptone, 0.5% yeast extract, 0.5% sodium chloride, and 1.5% agar) as described by Foulquie et al. (2003). Briefly, 20 µl of selected isolates suspensions (approximately 10^8 cfu/ml) was spotted onto sterile blood agar. Plates were incubated at 37 °C for 48 h and then observed for lyses zones formation around the colonies (positive reaction or β-hemolysis). The non-hemolytic reaction was recorded by observation of green-halo zones around the colonies (α-hemolysis) or was not produced any effect on the blood plates (γ-hemolysis). *S. aureus* was used as positive control (Foulquie et al. 2003).

Antagonistic effect of yeast isolates

For antimicrobial activity test, the isolated yeasts were inoculated into 40 ml YPD broth and incubated at 30ºC for 24 h and yeast suspensions were made. The clinically isolated pathogenic bacteria (*Salmonella typhi*, *E. coli* and *S. aureus*) were cultured in Mueller Hinton agar (MHA) at 37 °C for 24 h. MHA plates were prepared and inoculated (spread plated) with 0.1 ml using 0.5 McFarland of the pathogenic bacteria, left to dry for 15 min at 37 °C; 4 wells in each plate (with 5 mm diameter, 2 mm deep, and about 2 cm apart to minimize over lapping of zone) were bored using cork borer and filled with 1 ml of 10^8 cells/ml yeast suspension. The plates were incubated at 37 °C for 24 h, then the inhibition zones were measured (Khidhr 2014).

Antibiotics susceptibility test of probiotic yeast isolates

Antibiotic resistance test was carried out using Liofilchem antibiotic disks. Gentamicin, chloramphenicol, tetracycline, and ampicillin were used. The disks containing the antibiotics were placed onto the surfaces of YPDA plates that were inoculated with selected yeasts (10^8 cfu/ml). Inoculated plates were incubated at 37 °C for 48 h. Clear zone formed was measured using ruler (if there was) (Mohamed et al. 2020).

Molecular characterization of isolated probiotic yeasts

DNA extraction

The yeast isolates were first grown on YPD broth at 30 °C for 48 h. After incubation, 2 ml of each yeast cell suspension was taken and centrifuged at 15,000 rpm for 10 min. Then, the pellets were used to extract genomic DNA according to the protocol provided by NORGEN yeast genomic DNA isolation kit. Each extracted DNA was stored at 4 °C for further work.

Measurement of DNA concentration and purity

DNA concentration was measured using NanoDrop spectrophotometer. To the NanoDrop, 1.2 µl of tris EDTA elution buffer was used as a blank. The blank was removed by a tissue paper, and 1.2 µl sample was loaded (Prasad 2014). DNA concentration was measured in ng/µl. Agarose gel electrophoresis was also performed qualitatively determine the purity of isolated DNA.

Amplification of extracted DNA using polymerase chain reaction

The extracted genomic material of the D1/D2 domain of the 26S rRNA gene was amplified using polymerase chain reaction (PCR). This was done using NL1F and LS2R primers. The forward primer (NL1F) used was 5'

GCATAT CAAT AAGC GG AG GA AAAG 3' (Kurtzman and Robnett 1998), while the reverse primer (LS2R) used was 5' ATTCCCAAACAAC TCGACTC 3' (Cocolin et al.2000). The PCR mixture was prepared using Solis BioDyne 5xFIREPol^{R} Master Mix (Solis BioDyne, Riia, Tartu, Estonia, Europe) ready to load. The PCR reaction mix was prepared according to the recommended PCR reaction mix provided by 5 × FIREPol^{R} Master Mix ready to load. Reaction was set up for PCR carried out for 20 µl reaction volume in a 0.2 ml thin-walled PCR tube according to the manufacturers' guideline for 5 × FIREPol^{R} Master Mix. The temperature program and the cycle of reactions were as initial denaturation step at 95 °C for 5 min, followed denaturation at 95 °C for 30 s, primer annealing at 54 °C for 30 s, and primer extension at 72 °C for 2 min. The PCR was carried out for 30 cycles. Afterward, the amplicon was stored at − 20 °C for further work.

Detection of PCR product quality

After PCR reaction, amplification was checked using electrophoresis in 1% agarose gel in Tris–acetate-EDTA (TAE) buffer following the method used by Islam (2016). The gel was made in accordance with the manufacturer's instructions. About 12 µl aliquot of the PCR products was loaded into the individual wells of the gel. Ladders of size100 bp and 50 bp were used to ensure amplification of the desired DNA and the exact product sizes measured. The DNA bands were photographed with UV illuminator system.

Phylogenetic tree analysis

The yeast isolates of forward and reverse PCR products were sent to Netherlands and sequenced at Macrogen Company. After receiving sequenced PCR amplified products of yeast isolates, their sequence results of forward and reverse primers were edited and their consensus region were obtained using BioEdit. The 26S rRNA gene of the D1/D2 domain sequences was aligned with reference sequences showing sequence homology from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) database using the multiple sequence alignment program of Molecular Evolution Genetic Analysis (MEGA), version 7.0. The obtained sequence of their consensus region was submitted to BLAST (https://blast.ncbi.nlm.nih.gov) in order to find homologous sequences in GenBank. Phylogenetic analysis of gene sequence data was conducted using the neighbor-joining (NJ) method, and distances were computed by maximum composite likelihood method. The branching patterns were checked using bootstrap program in sets of 1000 bootstrap replicates. The BLAST algorithm was used to retrieve homologous sequences in GenBank (Kumar et al. 2016). Lastly, the sequencing findings were deposited in NCBI database and accession numbers were obtained for each isolate.

Data analysis

The data collected in this study were analyzed using SPSS software version 22. Means and standard deviations of the triplicates were evaluated using one-way analysis of variance (ANOVA) to determine the significance differences among the means followed by Duncan's multiple range tests. Finally, a variable with a p value less than or equal to 0.05 ($p \leq 0.05$) was considered to be statistically significant. One variable was analyzed independently at a time. BioEdit and MEGA7 bioinformatics softwares were used to analyze nucleotide sequences and phylogenetic tree, respectively.

Results

Isolation of probiotic yeasts from Ethiopian injera sourdough

In the current study, from 20 sourdough samples collected from Gondar and Bahir Dar cities, a total of 38 yeast isolates were screened based on their morphological characteristics. Among these, 10 yeast isolates (G1N1, G2N4, G3N1, G4N1, G7N1, G8N1, G8N2, B4N2, B4N5, and B6N3) that produced low or non-hydrogen sulfide were selected for further work.

Morphological characterization of yeast isolates

Concerning colony morphology, nine isolates had white colony color and one isolate, namely G3N1, had creamy colony color. Isolates G8N1 and G8N2 displayed irregular shape, while the rest were circular in shape. Some isolates (G8N1 and G8N2) were flat in colony elevation, whereas others were raised in elevation. Varied colony sizes were exhibited among the isolated colonies. All colonies displayed entire margin. Colonies of G8N1 and G8N2 had rough surface, while others had smooth surface properties. On the other hand, microscopic observations revealed that some of the studied yeast cells had oval shape, while some others were cylindrical in shape. In contrast, some had spherical in shape.

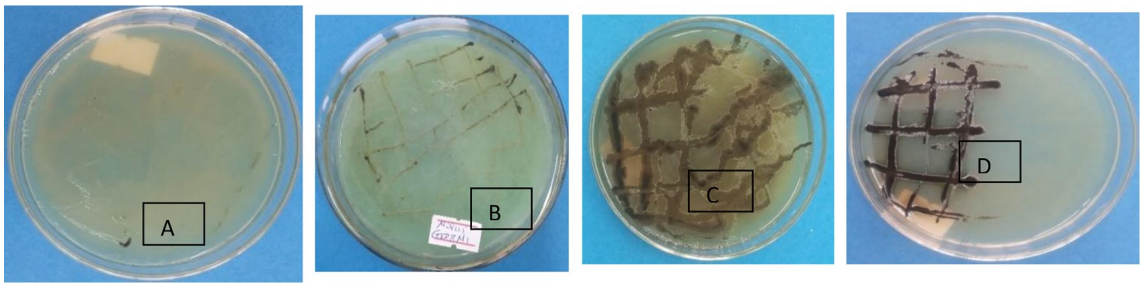

Fig. 1 Degree of hydrogen sulfide production by yeast isolates. **A** Non-hydrogen sulfide producers, **B** Low hydrogen sulfide producers, **C** Moderate hydrogen sulfide producers, and **D** High producers of hydrogen sulfide. Isolates G1N1 and G2N4 were found to be non-producers of hydrogen sulfide, while G3N1 and G8N1 produced light brown in color and B6N3 produced brown color. Hydrogen sulfide production associated with an off-flavor, unpleasant taste, texture, food spoilage, and poisoning can occur in food production. GI tract disorders of human have been attributed to the consumption of foods spoiled by yeasts that produced hydrogen sulfide. Yeast species that did not produce hydrogen sulfide and the ones that produce the range from light brown to brown under laboratory conditions are used as probiotics.

Hydrogen sulfide production test

Dehydrated Bacto Bismuth Sulfite Agar (BSA) was used for the selection of hydrogen sulfide producing yeast strains. The non-sulfide producing yeast strains had white colonies, while the hydrogen sulfide producers presented in various colony colors that ranged from light brown to black, depending upon the intensity of the production. From a total of 38 isolates, 10 isolates were low or non-producers of hydrogen sulfide as shown in Fig. 1a–c, while other yeast isolates were found to be high producers of hydrogen sulfide as shown in Fig. 1d. Isolate G1N1 and G2N4 were found to be non-producers of hydrogen sulfide, while G3N1 and G8N1 presented light brown colony color and B6N3 produced brown colony color. Therefore, all the five isolates were selected for further work.

Physiological characterization of probiotic yeast isolates

Temperature tolerance test

In test, eight yeast isolates (G1N1, G2N4, G3N1, G4N1, G7N1, G8N1, G8N2, and B6N3) were selected based on their better growth at 37 °C after 48 h incubation. Their growth was estimated in terms of their OD value at 600 nm, and some yeasts exhibited notable growth at 37 °C. From the result, it was found that isolate G4N1 had a maximum OD value (0.6667), while isolate B4N2 exhibited a minimum OD value (0.2400) (Table 1).

pH tolerance test

The growth at acidic pH of eight yeast isolates was evaluated using varied pH values: 2, 3, 4, and 5. From this test, five potential yeast isolates (G1N1, G2N4, G3N1, G8N1, and B6N3) were selected based on their acidic pH tolerance potential and better growth (Table 2). From the recorded results, isolate G3N1 gave a maximum OD value (0.4227) and isolate G8N2 had a minimum value (0.1400).

Bile salt tolerance test

Five yeast isolates were evaluated for bile salt tolerance test, and all yeast isolates exhibited good potential of bile salt tolerance and growth with different bile salt concentrations and selected for further work (Table 3). From these results, G2N4 gave a maximum OD value 0.8800 and G8N1 had a minimum OD value of 0.5493.

Osmotolerance test

Five yeast isolates were evaluated for osmotolerance test, and all isolates showed good potential to resist osmotic stress and grew well at different glucose concentrations (Table 4). Their tolerance and growth were recorded using their OD value at 600 nm. From this result, G8N1 had minimum OD values at 30% and 40% glucose concentration as compared to other yeast isolates.

Biochemical characterization of yeast isolates

Carbohydrate fermentation test

The fermentation capacity of five yeast isolates (G1N1, G2N4, G3N1, B6N3, and G8N1) was evaluated by supplying glucose, sucrose, galactose, maltose, and lactose as a carbon source. All yeast isolates fermented all sugars supplied except lactose.

Table 1 Temperature tolerance and growth of yeast isolates (OD value)

Isolates	Temperature	Growth value at different incubation periods		
		24 h	48 h	72 h
G1N1	25 °C	$(0.5500 \pm 0.06557)^{a}$	$(0.7967 \pm 0.06429)^{a}$	$(0.6367 \pm 0.01528)^{a}$
	30 °C	$(0.5400 \pm 0.02646)^{a}$	$(0.6400 \pm 0.01000)^{b}$	$(0.6400 \pm 0.01732)^{a}$
	37 °C	$(0.4433 \pm 0.03215)^{b}$	$(0.5733 \pm 0.02309)^{b}$	$(0.6067 \pm 0.01528)^{b}$
	42 °C	$(0.0233 \pm 0.00577)^{c}$	$(0.0200 \pm 0.00000)^{c}$	$(0.0200 \pm 0.00000)^{c}$
G2N4	25 °C	$(0.5067 \pm 0.2082)^{a}$	$(0.7200 \pm 0.03606)^{a}$	$(0.6200 \pm 0.04359)^{a}$
	30 °C	$(0.5267 \pm 0.6028)^{a}$	$(0.6800 \pm 0.07000)^{a}$	$(0.6100 \pm 0.05000)^{a}$
	37 °C	$(0.2633 \pm 0.2082)^{b}$	$(0.4000 \pm 0.02000)^{b}$	$(0.4233 \pm 0.01155)^{b}$
	42 °C	$(0.0267 \pm 0.0577)^{c}$	$(0.0200 \pm 0.01000)^{c}$	$(0.0233 \pm 0.00577)^{c}$
G3N1	25 °C	$(0.5500 \pm 0.7810)^{a}$	$(0.7833 \pm 0.06110)^{a}$	$(0.7233 \pm 0.05132)^{a}$
	30 °C	$(0.5300 \pm 0.1000)^{a}$	$(0.7233 \pm 0.04041)^{a}$	$(0.6667 \pm 0.02517)^{b}$
	37 °C	$(0.2967 \pm 0.3215)^{b}$	$(0.4200 \pm 0.02646)^{b}$	$(0.2967 \pm 0.01155)^{c}$
	42 °C	$(0.0233 \pm 0.1528)^{c}$	$(0.0200 \pm 0.01000)^{c}$	$(0.0200 \pm 0.01000)^{d}$
G4N1	25 °C	$(0.5600 \pm 0.02000)^{a}$	$(0.7900 \pm 0.04359)^{a}$	$(0.7167 \pm 0.01528)^{a}$
	30 °C	$(0.5900 \pm 0.01000)^{b}$	$(0.7700 \pm 0.01000)^{a}$	$(0.6867 \pm 0.01528)^{a}$
	37 °C	$(0.4867 \pm 0.01528)^{c}$	$(0.6667 \pm 0.03215)^{b}$	$(0.5800 \pm 0.03606)^{b}$
	42 °C	$(0.0500 \pm 0.01000)^{d}$	$(0.1700 \pm 0.02000)^{c}$	$(0.0200 \pm 0.01000)^{c}$
G7N1	25 °C	$(0.5167 \pm 0.00577)^{a}$	$(0.7467 \pm 0.00577)^{a}$	$(0.5800 \pm 0.07000)^{a}$
	30 °C	$(0.4933 \pm 0.02517)^{a}$	$(0.6633 \pm 0.01528)^{b}$	$(0.6167 \pm 0.00577)^{a}$
	37 °C	$(0.2767 \pm 0.00577)^{b}$	$(0.4200 \pm 0.00000)^{c}$	$(0.3200 \pm 0.00000)^{b}$
	42 °C	$(0.0233 \pm 0.00577)^{c}$	$(0.0200 \pm 0.01000)^{d}$	$(0.0200 \pm 0.01000)^{c}$
G8N1	25 °C	$(0.3500 \pm 0.01000)^{a}$	$(0.4333 \pm 0.01528)^{a}$	(0.3773 ± 0.01002)a
	30 °C	$(0.3773 \pm 0.00961)^{a}$	$(0.5867 \pm 0.03055)^{b}$	$(0.4407 \pm 0.00902)^{b}$
	37 °C	$(0.3923 \pm 0.01159)^{b}$	$(0.6433 \pm 0.00577)^{c}$	$(0.4667 \pm 0.01060)^{c}$
	42 °C	$(0.4100 \pm 0.02646)^{c}$	$(0.5300 \pm 0.07000)^{d}$	$(0.4333 \pm 0.01106)^{b}$
G8N2	25 °C	$(0.3367 \pm 0.02082)^{a}$	$(0.4633 \pm 0.03215)^{a}$	$(0.3737 \pm 0.00777)^{a}$
	30 °C	$(0.3920 \pm 0.01058)^{b}$	$(0.4833 \pm 0.05132)^{a}$	$(0.3900 \pm 0.01000)^{b}$
	37 °C	$(0.3993 \pm 0.01168)^{b}$	$(0.4633 \pm 0.05859)^{a}$	$(0.4167 \pm 0.00577)^{c}$
	42 °C	$(0.4027 \pm 0.02532)^{b}$	$(0.4233 \pm 0.06429)^{a}$	$(0.3893 \pm 0.00902)^{b}$
B4N2	25 °C	$(0.5333 \pm 0.01155)^{a}$	$(0.7933 \pm 0.01528)^{a}$	$(0.8533 \pm 0.03215)^{a}$
	30 °C	$(0.4933 \pm 0.03055)^{b}$	$(0.7300 \pm 0.02646)^{b}$	$(0.8133 \pm 0.02082)^{b}$
	37 °C	$(0.1300 \pm 0.01000)^{c}$	$(0.2400 \pm 0.01000)^{c}$	$(0.2467 \pm 0.00577)^{c}$
	42 °C	$(0.0167 \pm 0.00577)^{d}$	$(0.0200 \pm 0.00000)^{d}$	$(0.0167 \pm 0.01155)^{d}$
B4N5	25 °C	$(0.4433 \pm 0.04041)^{a}$	$(0.7067 \pm 0.04163)^{a}$	$(0.8067 \pm 0.07024)^{a}$
	30 °C	$(0.4400 \pm 0.06083)^{a}$	$(0.6833 \pm 0.06658)^{a}$	$(0.7733 \pm 0.06028)^{a}$
	37 °C	$(0.1300 \pm 0.01000)^{b}$	$(0.2500 \pm 0.01000)^{b}$	$(0.2400 \pm 0.01000)^{b}$
	42 °C	$(0.0133 \pm 0.00577)^{c}$	$(0.0133 \pm 0.00577)^{c}$	$(0.0133 \pm 0.00577)^{c}$
B6N3	25 °C	$(0.3567 \pm 0.01528)^{a}$	$(0.6867 \pm 0.03512)^{a}$	$(0.8067 \pm 0.01528)^{a}$
	30 °C	$(0.4667 \pm 0.01528)^{b}$	$(0.7300 \pm 0.02646)^{a}$	$(0.7967 \pm 0.02887)^{a}$
	37 °C	$(0.2767 \pm 0.01528)^{c}$	$(0.6400 \pm 0.01000)^{b}$	$(0.6900 \pm 0.01000)^{b}$
	42 °C	$(0.0267 \pm 0.00577)^{d}$	$(0.0200 \pm 0.01000)^{c}$	$(0.0167 \pm 0.00577)^{c}$

Values are mean ± SD of three replicates. Values followed by different superscripts are significantly different ($P \leq 0.05$). Values followed by same superscripts are not significantly different ($P \leq 0.05$)

Proteolytic activity test

To determine the proteolytic activity of yeast isolates, skim milk agar medium was used and colonies having a clear (halo) zone surrounding them were considered as strains with proteolytic activity. From this test, all the five yeast isolates: G1N1, G2N4, G3N1, G8N1, and B6N3, showed good proteolytic activity.

Table 2 pH tolerance and growth of yeast isolates

Isolates	pH value	Growth at different incubation periods (OD value)		
		24 h	48 h	72 h
G1N1	pH 2	$(0.1737 \pm 0.01150)^{a}$	$(0.3447 \pm 0.00351)^{a}$	$(0.4687 \pm 0.00850)^{a}$
	pH 3	$(0.4240 \pm 0.01200)^{b}$	$(0.6720 \pm 0.01200)^{b}$	$(0.7203 \pm 0.00643)^{b}$
	pH 4	$(0.4550 \pm 0.00755)^{c}$	$(0.7023 \pm 0.01595)^{b}$	$(0.8920 \pm 0.01153)^{c}$
	pH 5	$(0.4777 \pm 0.00551)^{d}$	$(0.6580 \pm 0.00200)^{c}$	$(0.7850 \pm 0.00200)^{d}$
G2N4	pH 2	$(0.2567 \pm 0.01050)^{a}$	$(0.3710 \pm 0.01300)^{a}$	$(0.4780 \pm 0.02400)^{a}$
	pH 3	$(0.4360 \pm 0.02000)^{b}$	$(0.5667 \pm 0.03150)^{b}$	$(0.7390 \pm 0.01513)^{b}$
	pH 4	$(0.4853 \pm 0.01060)^{c}$	$(0.6313 \pm 0.01582)^{c}$	$(0.7867 \pm 0.02050)^{b}$
	pH 5	$(0.5020 \pm 0.00100)^{c}$	$(0.6327 \pm 0.00351)^{c}$	$(0.7647 \pm 0.00451)^{c}$
G3N1	pH 2	$(0.2767 \pm 0.00751)^{a}$	$(0.4227 \pm 0.00252)^{a}$	$(0.4457 \pm 0.01050)^{a}$
	pH 3	$(0.4217 \pm 0.01650)^{b}$	$(0.6090 \pm 0.02600)^{b}$	$(0.5030 \pm 0.00400)^{b}$
	pH 4	$(0.4557 \pm 0.00153)^{c}$	$(0.6367 \pm 0.00153)^{c}$	$(0.5650 \pm 0.01700)^{b}$
	PH 5	$(0.5197 \pm 0.00551)^{d}$	$(0.6513 \pm 0.00603)^{c}$	$(0.5087 \pm 0.00058)^{c}$
G4N1	pH 2	$(0.0993 \pm 0.01007)^{a}$	$(0.2017 \pm 0.00723)^{a}$	$(0.1873 \pm 0.01007)^{a}$
	pH 3	$(0.1767 \pm 0.00577)^{b}$	$(0.3393 \pm 0.01419)^{b}$	$(0.3173 \pm 0.00635)^{b}$
	pH 4	$(0.4587 \pm 0.00850)^{c}$	$(0.8217 \pm 0.05064)^{c}$	$(0.5650 \pm 0.01700)^{c}$
	pH 5	$(0.4340 \pm 0.01100)^{d}$	$(0.6280 \pm 0.00700)^{d}$	$(0.5087 \pm 0.00058)^{d}$
G7N1	pH 2	$(0.0573 \pm 0.00814)^{a}$	$(0.2020 \pm 0.01500)^{a}$	$(0.1710 \pm 0.00608)^{a}$
	pH 3	$(0.2410 \pm 0.00529)^{b}$	$(0.3300 \pm 0.01000)^{b}$	$(0.2853 \pm 0.01102)^{b}$
	pH 4	$(0.3730 \pm 0.00500)^{c}$	$(0.6260 \pm 0.02600)^{c}$	$(0.5680 \pm 0.03300)^{c}$
	pH 5	$(0.3640 \pm 0.00900)^{c}$	$(0.5380 \pm 0.00200)^{d}$	$(0.4890 \pm 0.00900)^{d}$
G8N2	pH 2	$(0.0800 \pm 0.01000)^{a}$	$(0.1400 \pm 0.01000)^{a}$	$(0.1400 \pm 0.01000)^{a}$
	pH 3	$(0.2267 \pm 0.01528)^{b}$	$(0.3367 \pm 0.01528)^{b}$	$(0.3067 \pm 0.01528)^{b}$
	pH 4	$(0.2787 \pm 0.00451)^{c}$	$(0.4280 \pm 0.03300)^{b}$	$(0.3960 \pm 0.00300)^{c}$
	pH 5	$(0.2720 \pm 0.00300)^{c}$	$(0.3587 \pm 0.00153)^{c}$	$(0.3390 \pm 0.00300)^{d}$
B6N3	pH 2	$(0.1800 \pm 0.01153)^{a}$	$(0.3527 \pm 0.01079)^{a}$	$(0.4180 \pm 0.01572)^{a}$
	pH 3	$(0.3390 \pm 0.00500)^{b}$	$(0.5427 \pm 0.03550)^{b}$	$(0.5830 \pm 0.01500)^{b}$
	pH 4	$(0.3547 \pm 0.00153)^{c}$	$(0.6130 \pm 0.00300)^{b}$	$(0.7057 \pm 0.02450)^{c}$
	pH 5	$(0.3780 \pm 0.00700)^{d}$	$(0.5537 \pm 0.02150)^{c}$	$(0.6230 \pm 0.02200)^{d}$
G8N1	pH 2	$(0.1890 \pm 0.01300)^{a}$	$(0.3290 \pm 0.00100)^{a}$	$(0.3200 \pm 0.01400)^{a}$
	pH 3	$(0.2530 \pm 0.02000)^{b}$	$(0.3647 \pm 0.00681)^{b}$	$(0.3870 \pm 0.00200)^{a}$
	pH 4	$(0.2830 \pm 0.01386)^{c}$	$(0.3727 \pm 0.02450)^{b}$	$(0.3720 \pm 0.01000)^{b}$
	pH 5	$(0.2737 \pm 0.00551)^{c}$	$(0.3667 \pm 0.01250)^{b}$	$(0.3397 \pm 0.02650)^{b}$

Values are mean ± SD of three replicates. Values followed by different superscripts are significantly different ($P \leq 0.05$). Values followed by same superscripts are not significantly different ($P \leq 0.05$)

Lipolytic activity test

The lipolytic activity of yeast isolates was evaluated using tween 80 agar medium, and their growth pattern was observed on the plate. From this test, G1N1, G2N4, G3N1, and B6N3 showed good lipolytic activity, while G8N1 exhibited poor lipolytic activity.

Hemolytic activity test of yeast isolates

Five yeast isolates were evaluated for their hemolytic activity using blood agar media, and all yeast isolates were found to be non-pathogenic because they did not show any clear zone around the colony as compared to clear zone formed by positive control pathogenic bacteria, *S. aureus* (Fig. 2).

Table 3 Bile salt tolerance and growth test of yeast isolates

Isolates	Bile salt concentration	Growth at different incubation periods (OD value)	
		24 h	48 h
G1N1	0.1%	$(0.6850 \pm 0.01000)^{a}$	$(0.8937 \pm 0.00751)^{a}$
	0.3%	$(0.6587 \pm 0.00058)^{b}$	$(0.8240 \pm 0.02200)^{b}$
	0.5%	$(0.5280 \pm 0.00300)^{c}$	$(0.7387 \pm 0.03350)^{c}$
G2N4	0.1%	$(0.7037 \pm 0.01350)^{a}$	$(0.9120 \pm 0.02200)^{a}$
	0.3%	$(0.6760 \pm 0.00400)^{b}$	$(0.8800 \pm 0.05200)^{a}$
	0.5%	$(0.6117 \pm 0.01550)^{c}$	$(0.7777 \pm 0.04850)^{b}$
G3N1	0.1%	$(0.6937 \pm 0.01050)^{a}$	$(0.9187 \pm 0.00850)^{a}$
	0.3%	$(0.6587 \pm 0.00950)^{b}$	$(0.8387 \pm 0.02150)^{b}$
	0.5%	$(0.3530 \pm 0.01253)^{c}$	$(0.7227 \pm 0.01750)^{c}$
G8N1	0.1%	$(0.4767 \pm 0.02003)^{a}$	$(0.6990 \pm 0.01000)^{a}$
	0.3%	$(0.4083 \pm 0.01258)^{b}$	$(0.5493 \pm 0.02413)^{b}$
	0.5%	$(0.3483 \pm 0.02205)^{c}$	$(0.4777 \pm 0.01955)^{c}$
B6N3	0.1%	$(0.7187 \pm 0.02237)^{a}$	$(0.9363 \pm 0.04549)^{a}$
	0.3%	$(0.6607 \pm 0.02754)^{b}$	$(0.8350 \pm 0.02265)^{b}$
	0.5%	$(0.3647 \pm 0.02380)^{c}$	$(0.7180 \pm 0.02227)^{c}$

Values are mean ± SD of three replicates. Values followed by different superscripts are significantly different ($P \leq 0.05$). Values followed by same superscripts are not significantly different ($P \leq 0.05$)

Antimicrobial activity of yeast isolates

Antimicrobial activity of each yeast isolate was tested. All isolates displayed varied antimicrobial activities against different clinically isolated pathogenic bacteria (*S. aureus*, *E. coli,* and *S. typhi*) as compared to positive control, ciprofloxacin, as shown in Table 5.

Antibiotics susceptibility test

Five yeast isolates were evaluated for their antibiotic susceptibility test using different antibiotics (gentamicin, chloramphenicol, tetracycline, and ampicillin). All the five isolates were found to be resistant to all the tested antibiotics as no clear zone was formed around each tested antibiotic disks.

Molecular characterization of isolated probiotic yeasts

DNA extraction, measurement of DNA concentration, and purity

The genomic DNA of all selected and screened yeast isolates was successfully extracted using yeast genomic DNA extraction kit. The quantification (concentration of DNA) results for the selected isolates were 20, 31, 49, 63, and

Table 4 Osmotic effect test of yeast isolates

Isolates	Glucose concentration	Growth at different incubation periods (OD value)		
		24 h	48 h	72 h
G1N1	20%	$(0.2747 \pm 0.00451)^{a}$	$(0.3747 \pm 0.00451)^{a}$	$(0.4227 \pm 0.00950)^{a}$
	30%	$(0.2090 \pm 0.01300)^{b}$	$(0.2997 \pm 0.00351)^{b}$	$(0.3093 \pm 0.00231)^{b}$
	40%	$(0.1440 \pm 0.00800)^{c}$	$(0.2320 \pm 0.00100)^{c}$	$(0.2247 \pm 0.00551)^{c}$
	50%	$(0.1267 \pm 0.00513)^{d}$	$(0.2210 \pm 0.01100)^{c}$	$(0.1967 \pm 0.01242)^{d}$
G2N4	20%	$(0.2170 \pm 0.01600)^{a}$	$(0.3590 \pm 0.03000)^{a}$	$(0.4460 \pm 0.01700)^{a}$
	30%	$(0.1507 \pm 0.01750)^{b}$	$(0.3030 \pm 0.02166)^{b}$	$(0.3450 \pm 0.00900)^{b}$
	40%	$(0.1107 \pm 0.00751)^{c}$	$(0.2470 \pm 0.00200)^{c}$	$(0.2547 \pm 0.00058)^{c}$
	50%	$(0.0647 \pm 0.00751)^{d}$	$(0.2060 \pm 0.00400)^{d}$	$(0.2227 \pm 0.00850)^{d}$
G3N1	20%	$(0.2390 \pm 0.01400)^{a}$	$(0.3587 \pm 0.00252)^{a}$	$(0.4387 \pm 0.00351)^{a}$
	30%	$(0.1567 \pm 0.00651)^{b}$	$(0.3030 \pm 0.00600)^{b}$	$(0.3207 \pm 0.00451)^{b}$
	40%	$(0.1050 \pm 0.01600)^{c}$	$(0.2217 \pm 0.00351)^{c}$	$(0.2297 \pm 0.00651)^{c}$
	50%	$(0.0780 \pm 0.01400)^{d}$	$(0.1977 \pm 0.01079)^{d}$	$(0.1867 \pm 0.00577)^{d}$
G8N1	20%	$(0.2500 \pm 0.01000)^{a}$	$(0.2217 \pm 0.00850)^{a}$	(0.2680 ± 0.01750)a
	30%	$(0.1917 \pm 0.02219)^{b}$	$(0.1743 \pm 0.00709)^{b}$	$(0.1527 \pm 0.01266)^{b}$
	40%	$(0.1410 \pm 0.00854)^{c}$	$(0.1522 \pm 0.00490)^{c}$	$(0.1353 \pm 0.01484)^{b}$
	50%	$(0.0800 \pm 0.01000)^{d}$	$(0.1237 \pm 0.00950)^{d}$	$(0.1263 \pm 0.01600)^{b}$
B6N3	20%	$(0.3780 \pm 0.00700)^{a}$	$(0.3640 \pm 0.01150)^{a}$	$(0.3557 \pm 0.00950)^{a}$
	30%	$(0.3547 \pm 0.00153)^{b}$	$(0.3167 \pm 0.00300)^{b}$	$(0.3250 \pm 0.01539)^{b}$
	40%	$(0.3390 \pm 0.00500)^{c}$	$(0.1430 \pm 0.00100)^{c}$	$(0.1460 \pm 0.01800)^{c}$
	50%	$(0.1800 \pm 0.01153)^{d}$	$(0.0540 \pm 0.00400)^{d}$	$(0.0870 \pm 0.00200)^{d}$

Values are mean ± SD of three replicates. Values followed by different superscripts are significantly different ($P \leq 0.05$). Values followed by same superscripts are not significantly different ($P \leq 0.05$)

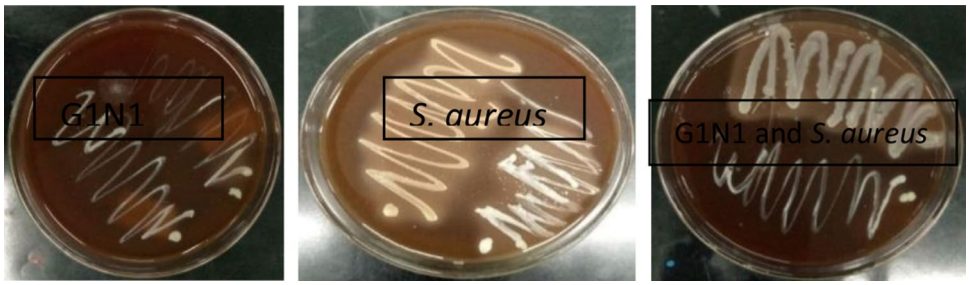

Fig. 2 Sample hemolytic activity test of yeast isolates. The selected yeast strains in the current study were non-hemolytic as opposed to hemolytic activity exhibited by strain of *S. aureus* that was used as positive control. If there were hemolytic activity, lyses zones around the colonies that grew on blood agar media were observed (positive reaction) similar to positive control. This proved that the isolated yeast strains under this study were non-pathogenic

Table 5 Antimicrobial activity of yeast isolates on clinically isolated pathogenic bacteria

Yeast isolates	Average inhibition zone (mm)		
	S. aureus	*E. coli*	*S. typhi*
G1N1	$(17.6667 \pm 0.57735)^{a}$	$(14.0000 \pm 1.00000)^{a}$	$(18.3333 \pm 0.57735)^{a}$
G2N4	$(14.0000 \pm 1.00000)^{b}$	$(16.6667 \pm 0.57735)^{b}$	$(18.0000 \pm 1.00000)^{a}$
G3N1	$(16.0000 \pm 1.00000)^{a}$	$(14.0000 \pm 1.00000)^{a}$	$(15.0000 \pm 1.00000)^{b}$
G8N1	$(16.0000 \pm 1.00000)^{a}$	$(18.3333 \pm .57735)^{c}$	$(16.6667 \pm 0.57735)^{b}$
B6N3	$(17.0000 \pm 1.00000)^{a}$	$(15.0000 \pm 1.00000)^{b}$	$(15.0000 \pm 1.00000)^{b}$
Ciprofloxacin	$(24.0000 \pm 1.00000)^{c}$	$(23.6667 \pm 1.52753)^{d}$	$(26.0000 \pm 1.00000)^{d}$

Values are mean ± SD of 3 replicates. Values followed by different superscripts are significantly different ($P \leq 0.05$). Values followed by same superscripts are not significantly different ($P \leq 0.05$)

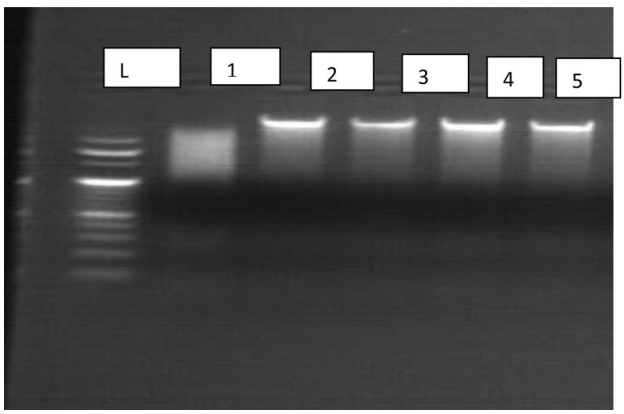

Fig. 3 Genomic DNA was successfully extracted from the five screened potential yeast isolates using yeast genomic DNA extraction kit. DNA concentration and purity were checked using NanoDrop and agarose gel electrophoresis. The quality and concentration of extracted genomic DNA fulfilled the criteria of good quality DNA because the absorbance (A260/A280) ratio was between 1.75 and 2. The qualitative estimation of DNA was done by running on agarose gel to determine the presence of pure DNA using agarose gel electrophoresis. Thus, DNA of good quality without any degradation and smear shows successful DNA extraction

55 ng/µl with a quality of 1.9, 1.8, 1.9, 2, and 1.75, at A260/A280 ratio of isolated yeast G1N1, G2N4, G3N1, G8N1, and B6N3, respectively. Qualitative estimation of DNA was done by running the DNA on agarose gel to determine the presence of pure DNA using agarose gel electrophoresis. DNA concentration and purity were checked using agarose gel electrophoresis which migrated along the lanes of wells 1, 2, 3, 4, and 5 indicating yeast genomic DNA of G1N1, G2N4, G3N1, G8N1, and B6N3, respectively, and together with lane L which is DNA ladder of 100 bp (Fig. 3).

PCR amplification

The extracted yeast genomic DNA was amplified using D1/D2 region of 26S large subunit ribosomal RNA gene using forward primer NL1 and reverse primer LS2. According to the PCR amplification result, approximate molecular size of the amplicons was 250 bp. Figure 4 shows size and purity of each potential probiotic yeast isolate PCR product with DNA ladder of 100 bp (L1) and 50 bp (L2).

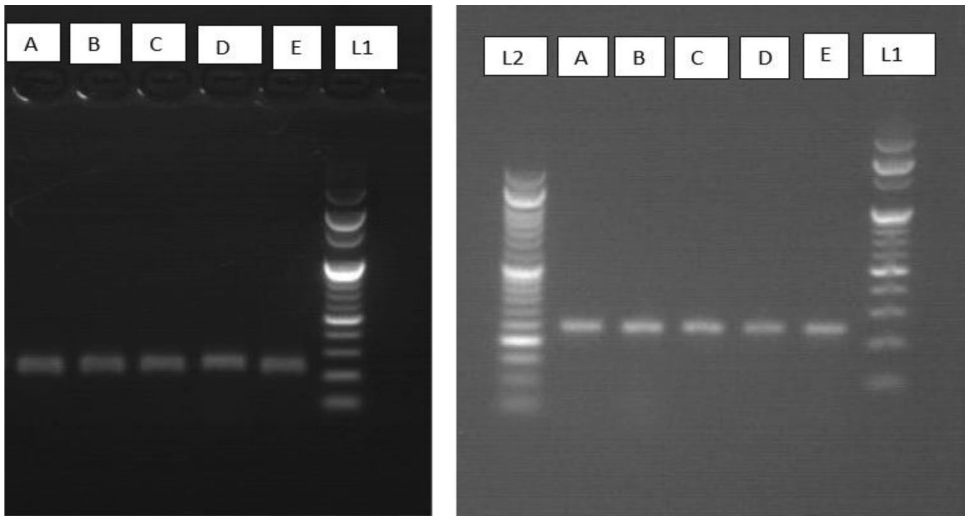

Fig. 4 Labeled A indicates GID1N1, B indicates GID2N4, C indicates GID3N1, D indicates GID8N1, E indicates AID6N3, L1 indicates 100 bp DNA ladder, and L2 indicates 50 bp DNA ladder. The acquired DNA bands from the five yeast isolates were observed to have a size of 250 bp

Table 6 Similarity of the isolates with known strain on NCBI blast results

Yeast Isolates	Percent of identity	Strain	Query value	*E* value	Accession number
G1N1	98.3%	*S. cerevisiae*	100%	8e−111	(KM521820.1)
G2N4	96.94%	*S. cerevisiae*	93%	1e−103	(KY073577.1)
G3N1	98.19%	*C. humilis*	90%	3e−105	(KM521815.1)
G8N1	97.81%	*P. kudriavzevii*	100%	1e−104	(MK044080.1)
B6N3	97%	*C. Humilis*	95%	2e−106	(KM521813.1)

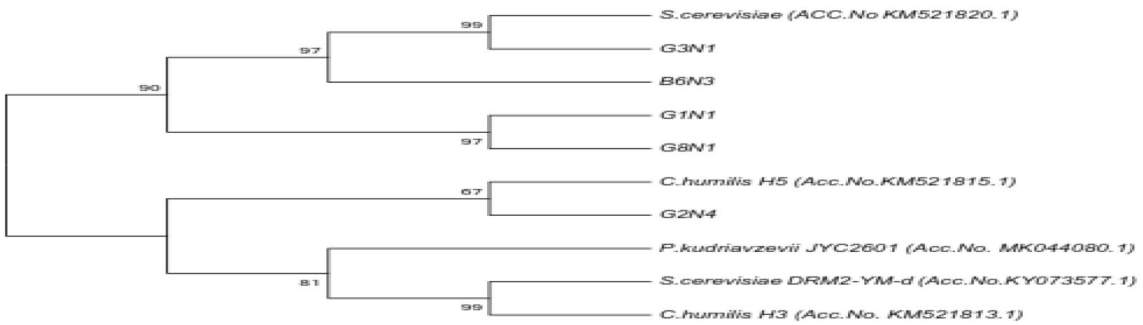

Fig. 5 Phylogenetic tree of the D1/D2 domain of 26S rRNA gene sequences of probiotic yeast isolates with related yeast species in NCBI database. Phylogenetic analysis of gene sequence data was conducted using the neighbor-joining (NJ) method of MEGA 7 software, and distances were computed by maximum composite likelihood method. The branching patterns were checked using bootstrap program in sets of 1000 bootstrap replicates. Each the strain has the following accession numbers. B6N3 (ACC. NO. OP942415); G1N1 (ACC. NO. OP942416); G2N4 (ACC. NO. OP942417); G3N1 (ACC. NO. OP942418); and G8N1 (ACC. NO. OP942419)

Phylogenetic tree analysis

PCR products of genomic DNA of five yeast isolates were sequenced at Macrogen Company, Netherlands. After sequencing, PCR amplified products of each yeast isolate, their sequence products were edited and their consensus regions were obtained using BioEdit and MEGA7 bioinformatics software. Probiotic isolates G1N1, G2N4, G3N1, G8N1, and B6N3 were found to be 98.3%, 96.94%, 98.19%, 97.81%, and 97% identical to *S. cerevisiae* (Acc. No.KM521820.1), *S. cerevisiae* (Acc.No.KY073577.1), *Candida humilis* (Acc.No.KM521815.1), *Pichia kudriavzevii* (Acc.No.MK044080.1), and *Candida humilis* (Acc. NoKM521813.1), respectively (Table 6).

Finally, the phylogenetic tree of sequences obtained from this study and that of closely related sequences from GenBank (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were constructed. The D1/D2 domain sequences were

aligned, and the phylogenetic relationship of these yeast strains is displayed by a maximum composite likelihood-based neighbor-joining tree (Fig. 5). The probiotic isolates were also given GenBank accession number (ACC. NO.) as follows: B6N3 (ACC.NO.OP942415); G1N1 (ACC. NO.OP942416); G2N4 (ACC.NO. OP942417); G3N1 (ACC. NO.OP942418); and G8N1 (ACC. NO.OP942419).

Discussion

Currently, some yeasts species like *S. cerevisiae var. boulardii* have been considered as novel probiotics. *S. cerevisiae var. boulardii* has been widely applied in food industry and health as it has beneficial effects such as improving intestinal conditions (Moradi et al.2018). There is an immense interest by microbiologists to isolate, screen, and explore other yeasts from fermentation products and subsequent evaluations of their specific probiotic properties.

From 20 collected and processed injera sourdough samples, a total of 38 yeast isolates were screened based on their colony morphological characteristics. The majority isolates of had white and few had creamy colony color. Some had irregular colony shape, while others displayed circular shape. Some of them had flat shape, while others were raised in elevation. In terms of colony size, some were large, while others were medium in size. Some colonies had rough surface, while others displayed smooth surface properties. All colonies had entire margin.

According to microscopic observation tests, some of the yeast cells had oval shape, while others displayed cylindrical shape and the remaining had spherical shaped structure. All isolates were found to be reproduced by budding. These results were in consistent with the previous findings by Walker et al. (2017), indicating that most of these kinds of morphological characteristics are the feature of *Saccharomyces* species and related yeasts.

In the current study, from 38 yeast isolates, 28 of them were found to produce hydrogen sulfide with the degree of producing black colony color in BSA media. But, the remaining10 isolates were considered as low or non-producers of hydrogen sulfide. This was confirmed by the production of light brown to brown color on BSA media by the 10 yeast isolates. In this study, the 10 yeast isolates that passed hydrogen sulfide production screening test were preserved for further tests. From this, the finding in the current study is similar to the research report by Yuma (2020). Hydrogen sulfide production associated with an off-flavor, unpleasant taste, texture, food spoilage, and poisoning can occur in food production. GI tract disorders of human have been attributed to the consumption of foods spoiled by yeasts that produced hydrogen sulfide. Yeast species that did not produce hydrogen sulfide and the ones that produce the range from light brown to brown under laboratory conditions are used as probiotics and do not produce bad and unwanted texture and flavors in food industry.

One of the criteria for selecting probiotic yeast includes their ability to tolerate and grow at human body temperature (37 °C). The isolated yeasts were selected based on their growth concentration of OD value at 37 °C (Rajkowska 2010). Most yeast isolates exhibited higher growth at 48 h, and a smooth decline in their growth was exhibited towards 72 h incubation. Therefore, 48 h was the best incubation period for the selected potential yeast isolates and from 10 yeast isolates, eight of them (G1N1, G2N4, G3N1, G4N1, G7N1, G8N1, G8N2, and B6N3)) exhibited high growth value at 48 h incubation period. Among the isolated yeasts, G4N1, G8N1, B6N3, and G1N1 exhibited maximum growth value at 37 °C with their OD value of 0.6667 ± 0.03215, 0.6433 ± 0.00577, 0.6400 ± 0.01000, and 0.5733 ± 0.02309, respectively. On the other hand, isolates G8N2, G3N1, G7N1, and G2N4 had medium growth OD value at 37 °C of 0.4633 ± 0.05859, 0.4200 ± 0.02646, 0.4200 ± 0.00000, and 0.4000 ± 0.02000, respectively. While isolates G8N1 and G8N2 also grew at 42 °C with their OD value of 0.5300 ± 0.07000 and 0.4233 ± 0.06429, respectively, this result was similar to the research report of Moradi et al. (2018). On the other hand, B4N5 and B4N2 had minimum growth at 37 °C of 0.2500 ± 0.01000 and 0.2400 ± 0.01000, respectively, and they were not selected for further tests. But the growth of G1N1 isolate was no statistically different at 30 °C and 37 °C. On the other hand, G8N1 gave a maximum growth yield of 0.6433 ± 0.00577 at 37 °C and G8N2 did not show any statistical significance difference between all temperature factors.

Before reaching the intestinal tract, probiotic yeasts must survive transit through the stomach and exposure to gastric acid constituents, which is a primary defense mechanism against most ingested microorganisms. The promising activities of yeasts as well as their ability to survive during the passage through the human GI tract, tolerating exposures to low pH and bile salts, have drawn attention to their possible use as probiotics (Lourens and Viljoen 2001). The isolated yeasts exhibited the ability to resist acidity, and they were differed in ability to resist low pH (pH 2). Isolate G3N1 had highest growth at 48 h with OD value of 0.4227 ± 0.00252, and other isolates (G2N4, B6N3, G1N1, and G8N1) also tolerated lower acidity (pH 2) with their growth OD value of 0.3710 ± 0.01300, 0.3527 ± 0.01079, 0.3447 ± 0.00351, and 0.3290 ± 0.00100, respectively. This result is in agreement with research report of Chen et al. (2010) that describes some yeast isolates can tolerate and grow at pH 2. Isolates G7N1, G4N1, and G8N2 exhibited lowest survival and growth at pH 2 with OD value of 0.2020 ± 0.01500, 0.2017 ± 0.00723, and 0.1400 ± 0.01000, respectively, and were not selected for further tests. The optimum growth

values of yeast isolates in different pH values were evaluated, and most yeast isolates exhibited an optimum growth at pH 4. The yeast isolates G1N1, G8N2, and B6N3 were not significantly different ($P \leq 0.05$) at pH 3 and pH 4. The other yeast isolates (G4N1 and G7N1) were significantly different ($P \leq 0.05$) in all pH values, and they had optimum growth at pH 4. In addition to this, G2N4 and G3N1 were not significantly different at pH 4 and pH 5 and their optimum growth was at pH 4 and pH 5.

Microorganisms that survive in the acidic condition of the stomach also have to survive in intestinal secretion and the bile salts in the duodenum. It is synthesized in the pericentral hepatocytes of the liver, stored, and concentrated in the gall bladder interdigestively; concentration used for the screening of a resistant probiotic strain is 0.3% w/v (Khidhr 2014). As shown in Table 3, isolates G2N4, G3N1, G1N1, and B6N3 had shown better ability to tolerate 0.3% bile salt concentration than that of isolate G8N1 and had good growth even in 0.5% bile salt concentration. This result is in concurrent with the research report by Chenet al. (2010) that states yeasts have good tolerance and growth rate from 0.1%, 0.3%, and 0.5% of bile salt concentrations. Even though G8N1 had lower growth rate as compared to other isolates, it can tolerate and grow efficiently at 0.3% bile salt concentration. All the isolates showed different abilities of tolerance to bile salts. Generally, the growth rate of all yeast isolates was statistically significant at 0.1%, 0.3%, and 0.5% bile salt concentration except G2N4 which was not statistically significant at 0.3% and 0.5% bile salt concentrations at $p \leq 0.05$.

All isolated yeasts showed good tolerance to different osmotic effects of 20%, 30%, 40%, and 50% w/v glucose concentration. Among all the isolated yeasts, G8N1 exhibited lower tolerance at 40% and 50% of glucose concentrations, respectively. It indicates that probiotic yeasts that are administered to the stomach in the form of food are not affected by any osmotic effect in the stomach. All the five isolates tolerated and grew at 20% and 30% glucose concentrations very well, and they can proliferate in high osmotic effect. Generally, the growth and tolerance of all yeast isolates were statistically significantly different in all glucose concentrations at $p \leq 0.05$.

The selected five yeast isolates were tested for their ability to ferment carbohydrates. All isolates fermented the given carbohydrates except lactose and classified as sugar fermenters. The fermentation was determined visually by monitoring the color change with gas formation in Durham tubes. Isolates G1N1, G2N4, G3N1, and B6N3 were able to ferment glucose, galactose, and sucrose very well with the production of carbon dioxide gas, they fermented maltose without gas production, and only color change of the phenol red media to yellow color of the YPD medium broth was exhibited, while G8N1 fermented glucose, galactose, sucrose, and maltose without gas production in Durham tubes. This result was more or less similar to the research report of Khidhr (2014) and Yuma (2020). Sugar fermenter probiotic yeasts produce organic acids, short chain fatty acids, butyric acid, and ethanol thereby reducing the pH of their environment into an acidic condition which creates an unsuitable environment for pathogenic organisms in the GI tract because most pathogenic microorganisms prefer to grow best around neutral pH and yeasts themselves tolerate this acidic condition.

Proteolytic activity of yeast isolates was evaluated using skim milk agar medium, and five yeast isolates displayed good proteolytic activity. This may be due to the production of protease enzyme by the isolates into the extracellular environment. They formed clear zones around their colonies, and this indicates that probiotic yeast isolates possessed protease enzyme that degrade casein protein found in skim milk. On the other hand, this proteolytic activity can be used for degrading non-digestible food ingredients in the stomach and proteins changed to amino acids. Thus, these isolated yeasts can solve the problem of protein scarcity in the developing countries.

On the other hand, lipolytic activity of isolates was evaluated to know the ability of each isolate to degrade lipids to glycerol and short chain fatty acids which are used to produce butyric acid, acetic acid, and ethanol that inhibit the growth of pathogenic microorganisms. And also by observing their growth pattern on tween 80 media, four yeast isolates exhibited lipolytic activity. Isolated yeasts could grow on the media in scattered form, and it indicates that the isolated yeasts possessed lipase enzyme that degrade lipid found in tween 80 media. G8N1 did not show any lipolytic activity. This may be due to lack of lipase enzyme to utilize tween 80 media. This result was in concurrent with the research report by Moradi et al. (2018) who stated that most yeast isolates had proteolysis and lipolysis activity which have been found to be the main biochemical reactions in probiotic microorganisms to obtain amino acids, short chain fatty acids, esters, and other food components from degradation of protein and lipids.

The yeast strains were also tested for hemolytic activity. The non-hemolytic reaction was recorded in all the five yeast isolates, and this result was in concurrent with the research report of Ragavan et al. (2017). This means that the hemolysis of blood agar media was not exhibited as opposed to hemolytic activity exhibited by strain of *S. aureus* that was used as positive control. If there were hemolytic activity, lyses zones around the colonies that grew on blood agar media was observed (positive reaction) similar to positive control. None of the isolates exhibited hemolysis (clear blood lysis zones) in blood agar plates which proved that the isolated yeast strains under this study were non-pathogenic.

In the current study, five yeast isolates were checked for antimicrobial activity against different clinically isolated pathogenic bacteria. All isolated yeasts displayed different inhibition zones against different pathogenic bacteria. G8N1, G3N1, G1N1, and B6N3 had highest inhibition zone of 16.0000 ± 1.00000, 16.0000 ± 1.00000, 17.6667 ± 0.57735, and 17.0000 ± 1.00000, respectively, on *S. aureus* as a result of software analysis because they were not significantly different, while G2N4 had a minimum inhibition zone of 14.0000 ± 1.00000 against *S. aureus*. On the other hand, G8N1 had maximum inhibition zone (18.3333 ± 0.57735) against *E. coli* and G2N4 and B6N3 had a medium inhibition zone of 16.0000 ± 1.00000 and 15.0000 ± 1.00000, respectively. But, G1N1 and G3N1 gave a minimum OD value and the same inhibition zone of 14.0000 ± 1.00000 on *E. coli* pathogenic bacteria. In this result, the antimicrobial effect of all yeast isolates on *E. coli* was more effective as compared to the research reported by Yuma (2020). G1N1 and G2N4 had a maximum inhibition zone of 18.3333 ± 0.57735 and 18.0000 ± 1.00000 on *S. typhi*, respectively, and G3N1, G8N1, and B6N3 had a medium inhibition zone of 15.0000 ± 1.00000, 16.6667 ± 0.57735, and 15.0000 ± 1.00000 on *S. typhi*, respectively, as compared to among yeast isolates. The antimicrobial effect of yeast isolates on *S. typhi* was more or less in line with the research report of Khidhr (2014). Ciprofloxacin was used as a positive control, and it had inhibition zone against *S. aureus*, *E. coli,* and *S. typhi* with 24.0000 ± 1.00000, 23.6667 ± 1.52753, and 26.0000 ± 1.00000, respectively. Generally, all yeast isolates showed effective antimicrobial activity on different clinically isolated pathogenic bacteria as compared to the positive control of ciprofloxacin. Antimicrobial activities in probiotic yeasts are desirable properties to all probiotic microorganisms. It indicates that it is important for the probiotic yeast strain to have a competitive advantage and prevent the colonization of the intestine by pathogenic bacteria.

Antibiotic susceptibility test of potential probiotic isolates is also considered as an important selection criterion for their potential probiotic status. All the five isolates were evaluated for their antibiotic susceptibility test, and all isolated yeasts were resistant to different antibiotics (gentamicin, chloramphenicol, tetracycline, and ampicillin). No any clear zone around antibiotics disks was exhibited in this study, and this indicates that there is no any effect on probiotic yeast when peoples take bacterial antibiotics.

The quality and concentration of extracted genomic DNA in the current study fulfilled the criteria of good quality DNA because the absorbance (A260/A280) ratio was between 1.75 and 2 (Fig. 3). As expected, the acquired DNA bands from the five yeast isolates were observed to have a size of 250 bp as shown in Fig. 4. On the other hand, molecular nucleotide sequence was analyzed using BioEdit bioinformatics software and their consensus region was blasted on NCBI and compared with highly similar matched yeast species based on percent of identity. G1N1 was matched with *S. cerevisiae* (KM521820.1) with its percent of identity 98.3%, e value 8e-111, and query of 100%. G2N4 was matched with *S. cerevisiae* (KY073577.1) with its percent of identity 96.94%, e value 1e-103, and query of 93%. G3N1 was similar to *C. humilis* (KM521815.1) with its percent of identity 98.19%, e value 3e -105, and query of 90%. G8N1 was similar to *P. Kudriavzevii* of accession number (MK044080.1) with its percent of identity 97.81%, e value 1e-104, and 100% query. This yeast strain is confirmed from the information reported by Staniszewski and Kordowska (2021) that states the probiotic strain *P. kudriavzevii* was used to produce fermented cereal-based food. It plays a role in increasing antioxidant activity, cholesterol reduction, and enhancing a variety of flavor compounds in fermented foods. B6N3 was found to be matched with *C. humilis* of accession number (KM521813.1) with its percent of identity 97%, e value 2e-106, and 95% query. Among the five yeast isolates G1N1 was more similar to *S. cerevisiae* (KM521820.1) as compared in terms of its percent of identity. The details are presented in Table 6. Among the five yeast isolates G1N1 and G2N4 were similar to *S. cerevisiae*, which has an interesting beneficial effect on the nutritional value of foods of plant origin since it synthesizes folates and eliminates phytates and other antinutrients. Enzyme phytase produced by this yeast could enhance the bioavailability and absorption of essential minerals such as iron, zinc, magnesium, and phosphorus. Another advantage of *S. cerevisiae var. boulardii* may be its antimicrobial properties to inhibit the growth of *E. coli*, *S. typhi,* and *S. aureus* as well as its abilities to decompose mycotoxins such as aflatoxins, patulin, ochratoxin A, and other fungal toxins (Staniszewski and Kordowska 2021).

Finally in this study, phylogenetic tree of five isolates was constructed using MEGA 7 bioinformatics software to know the evolutionary relationships between the isolates and GenBank deposited similar strains. All five yeast isolates had one common ancestor, and each isolates were evolutionary related to their aligned matches of well-known yeast strains from NCBI GenBank (Fig. 5).

Conclusion

Ethiopia is rich in fermented foods, in which yeasts are the predominant microorganisms found in traditional fermented sourdough. In this study, potential probiotic yeasts were isolated, screened, and characterized from Ethiopian fermented injera sourdough. These yeasts could grow at 37 °C and tolerate the low pH of gastric acid and different bile salt concentrations. Therefore, the study provides bioavailability of

food nutritional value of fermented foods and it can increase fermentation process, food production time, and beneficial health effects of GI tract. It is recommended that whole genome sequencing should be done for further characterization of the isolated probiotic yeasts. The data obtained in this study suggest that a possible use of *S. cerevisiae*, *C. humilis,* and *P. kudriavzevii* can be potential probiotics that are isolated from Ethiopian fermented injera sourdough.

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Authors' contributions NM performed the experiments as part of his master's thesis work. All this work was carried out under the supervision of TG and TMJ. TMJ helped in editing the manuscript. All authors read and approved the final manuscript.

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Data availability The data used to support the findings of this study are included within the article. The nucleotide sequence data are available in the GenBank (NCBI) databases under the accession numbers of OP942415 (for B6N3), OP942416 (for G1N1), OP942417 (for G2N4), OP942418 (for G3N1), and OP942419 (for G8N1).

Declarations

Conflicts of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals We confirm that the research does not involve human participants and/or animals.

Informed consent Since the study does not involve any human participants, informed consent is not applicable for the study.

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